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Armored RNA as Virus Surrogate in a Real-Time Reverse Transcriptase PCR Assay Proficiency Panel

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In recent years testing responsibilities for high-consequence pathogens have been expanded from national reference laboratories into networks of local and regional laboratories in order to support enhanced disease surveillance and to test for surge capacity. This movement of testing of select agents and high-consequence pathogens beyond reference laboratories introduces a critical need for standardized, noninfectious surrogates of disease agents for use as training and proficiency test samples. In this study, reverse transcription-PCR assay RNA targets were developed and packaged as armored RNA for use as a noninfectious, quantifiable synthetic substitute for four high-consequence animal pathogens: classical swine fever virus; foot-and-mouth disease virus; vesicular stomatitis virus, New Jersey serogroup; and vesicular stomatitis virus, Indiana serogroup. Armored RNA spiked into oral swab fluid specimens mimicked virus-positive clinical material through all stages of the reverse transcription-PCR testing process, including RNA recovery by four different commercial extraction procedures, reverse transcription, PCR amplification, and real-time detection at target concentrations consistent with the dynamic ranges of the existing real-time PCR assays. The armored RNA concentrations spiked into the oral swab fluid specimens were stable under storage conditions selected to approximate the extremes of time and temperature expected for shipping and handling of proficiency panel samples, including 24 h at 37°C and 2 weeks at temperatures ranging from ambient room temperature to −70°C. The analytic test performance, including the reproducibility over the dynamic range of the assays, indicates that armored RNA can provide a noninfectious, quantifiable, and stable virus surrogate for specific assay training and proficiency test purposes.

National and international efforts to enhance early disease detection and to increase diagnostic capacity have stimulated the formation of laboratory networks within and between public health, animal health, and plant health arenas. Key to the success of these laboratory networks is the use of standardized procedures and assays in all of the associated laboratories, which in turn is reliant on specific training programs as well as a demonstrated proficiency of laboratory workers to perform the assays in question. For molecular biology-based assays, evaluations of proficiency test practices have identified analytic errors associated with all stages of the testing process as well as errors specific to the physical setup of individual laboratories, emphasizing the need for on-site proficiency testing (2, 3, 11, 15, 16). There are, however, biosecurity risks associated with the distribution of live agents for training or proficiency test purposes, as documented by the inadvertent global distribution of a pandemic strain of influenza A/H2N2 virus in a public health laboratory proficiency panel during early 2005. Within the veterinary community, the U.S. Department of Agriculture (USDA) has initiated the transfer of real-time PCR-based assays for selected high-economic-impact veterinary pathogens to state and university diagnostic laboratories within a national animal health laboratory network. Technical training for sample handling and testing of live virus within network laboratories by the use of on-site equipment and facilities is not feasible due to the strict select agent and biocontainment control necessary for the foreign animal disease agents. For the PCR-based foreign animal disease assays, all of which have been directed at RNA virus targets to date, the distribution of live virus has been avoided by using RNA mimics (2) and chemically inactivated virus (1, 14) as assay positive controls and proficiency test samples, respectively. Mimics provide an RNA template suitable for the evaluation of PCR amplification and detection steps, but since they are added to the assay immediately before the reverse transcription (RT) step, mimics cannot be used to measure the efficiency of sample processing, critical RNA extraction steps, and the potential for sample cross-contamination. Chemical treatment carries the risk of incomplete virus inactivation, template RNA denaturation from sample RNase activity, the potential for residual chemical inhibition of the PCR, and the relative instability of the target RNA. As a practical consideration, chemical treatment additionally requires extremely expensive and time-consuming in vivo and in vitro testing prior to distribution to ensure that no viable virus remains. With the movement of high-consequence pathogen and select agent detection assays beyond the limited number of federal laboratories, the design and establishment of safe, complete, and reliable training, quality control, and proficiency samples have become critical needs.

Armored RNA is a noninfectious and quantifiable synthetic substitute for live or chemically inactivated RNA virus that was originally designed for use as a calibration standard or internal assay control for reverse transcription real-time or quantitative RT-PCR (qRT-PCR) (10). The packaged or armored RNA is resistant to RNase digestion. Template RNA can be freed from the protective coat proteins by using heat or chemical RNA extraction procedures, which make the RNA available as a target for reverse transcription in the same manner as viral

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RNA is freed from an intact virion. The utility of armored RNA as an assay standard has been documented in clinical applications, where precise quality control and assay reproducibility are critical (4, 8, 17). Armored RNA has been shown to be stable in plasma for 6 months at −20°C and 2 months at 4°C (10) and to function equally well with a range of sample matrices, including nasopharyngeal secretions, lavage fluids, plasma, feces, and water (4). Armored RNA design can accommodate more than 1,200 bp of sequence information, which allows PCR targets, including the primer and probe sites, for multiple agents to be included in one RNase-resistant package. In the study reported here, assay-specific armored RNA was added directly to assay-appropriate clinical sample matrices to provide a noninfectious and quantifiable target for four high-consequence foreign animal disease RNA viruses: foot-and-mouth disease virus (FMDV); classical swine fever virus (CSFV); vesicular stomatitis virus, New Jersey serogroup (VSV-NJ); and vesicular stomatitis virus, Indiana serogroup (VSV-IND). The virus surrogate armored RNA was designed to provide a means of evaluating all steps for each of the four selected qRT-PCR assays, including sample handling (potential cross-contamination), RNA extraction and recovery, reverse transcription, primer binding, amplification, probe binding, and detection.

MATERIALS AND METHODS

Armored RNA. A 462-bp nucleic acid sequence was designed to include PCR targets for four foreign animal disease virus assays that have recently been transferred from the USDA Foreign Animal Disease Laboratory on Plum Island, N.Y., to designated veterinary diagnostic laboratories nationally. The targets included the forward and reverse primer sites, short flanking regions, and probe-binding sites previously published or described for each of the four qRT-PCR assays currently being validated by the USDA for enhanced foreign animal disease detection. The target sequence for classical swine fever virus is 102 bp in length (12, 13), the FMDV target is 123 bp (6), and the VSV-NJ and VSV-IND targets are 66 bp each (the sequences were kindly provided by Luis Rodriguez, Agricultural Research Service, USDA). A 20- to 40-bp "spacer" sequence was placed between each viral target. The spacer sequences, each of which included a unique restriction enzyme site, were designed into the RNA template to allow the flexibility to alter or substitute additional sequences at a later time, if required. The three restriction sites include nucleic acid sequences sensitive to digestion with restriction enzymes BglI, MluI, and PstI. The incorporation of specific restriction sites was included to allow the use of direct sequence analysis or restriction fragment analysis to distinguish the PCR products generated by the armored RNA from those generated by native virus, should laboratory contamination be suspected. Two additional restriction sites, HindIII and BamHI, were included at each end of the custom design to allow insertion of the template into a plasmid for cloning and armored RNA production. Once it was designed, the RNA template was commercially synthesized de novo (Sigma-Aldrich Inc., St. Louis, Mo.) and was provided as a synthetic gene to be commercially packaged as the final armored RNA product (Armored RNA Quant; Ambion, Inc., Austin, Tex.). Briefly, the patented technology uses an in vitro packaging reaction for encapsulation of the synthetic gene by combining purified *Escherichia coli* bacteriophage MS2 coat protein dimers and RNA transcribed in vitro. Armored RNA particles are harvested by RNase treatment, followed by conventional protein purification steps (10).

Real-time RT-PCR assays. The assay reagents, including qRT-PCR primers, qRT-PCR hydrolysis probes, deoxynucleoside triphosphates, PCR enzymes and buffers, plus the assay-specific standard operating procedures for the detection of CSFV (12, 13), FMDV (6), and the vesicular stomatitis viruses (kindly provided by Luis Rodriguez), were obtained from the USDA Foreign Animal Disease Diagnostic Laboratory at Plum Island, N.Y. The four qRT-PCR assays were specifically chosen based on their prior designation by USDA for use in the national animal health laboratory network. The nucleic acid extraction procedures used to test the armored RNA included a standard phenol-chloroform protocol, a commercially available silica-based column kit (RNeasy; QIAGEN, Inc., Valencia, Calif.), a prototype commercial silica-based column kit (Ambion, Inc., Austin, Tex.), and magnetic bead-based extraction (MagMax; Ambion, Inc.). All kit extraction and assay steps were performed as recommended by the previously referenced assay developer or extraction kit manufacturer. Briefly, for the phenol-chloroform extraction, 250 μl of sample was added to 750 μl of reagent (TRIzol LS; Invitrogen, Inc., Carlsbad, Calif.) in a phase separation tube (Phase Lock Gel; Brinkmann Inc., Westbury, N.Y.), and according to the manufacturer-recommended centrifugation and wash procedures, RNA was recovered in a final 18-μl volume. Both column extractions used manufacturer-recommended sample lysis, RNA binding, wash, and RNA elution buffers with sample starting and RNA recovery volumes of 140 μl and 40 μl, respectively (RNeasy; QIAGEN) or 200 μl and 50 μl, respectively (Ambion, Inc.). The magnetic bead procedure was performed in a 96-well format with sample start and RNA recovery volumes of 50 μl and 25 μl, respectively. The qRT-PCR was performed for 45 PCR cycles for each assay by using Smartcycler II (Cepheid, Inc., Sunnyvale, Calif.) equipment platforms with the paired software for qRT-PCR in either a single-target format (FMDV and CSFV assays) or a multiplex format (VSV-NJ plus VSV-IND assays).

Proficiency panel. Bovine oral swab specimens were collected individually and placed into 5 ml of viral transport medium (HEPES-buffered minimal essential medium containing 100 μg/ml gentamicin and 0.5 μg/ml amphotericin B) and transported to the laboratory, where the swab was removed and the remaining sample was stored at −20°C until it was used. In order to realistically mimic diagnostic case material, the samples were not centrifuged or filtered to remove saliva, epithelial cells, bacteria, feed, or other debris. The custom-designed armored RNA was spiked in serial 10-fold dilutions into pooled swab fluid at dilutions that targeted the previously reported assay detection limits and qRT-PCR assay target ranges by using cycle threshold (C_T) values between 18 and 45. To monitor the stability of the armored RNA, pooled swab samples were spiked with armored RNA diluted 10^{-3}, 10^{-4}, and 10^{-5} from the stock concentration of 5×10^9 particles per μl to approximate the mid- to endpoint detection limits of all four assays. The same pooled swab fluid specimen containing no armored RNA was tested in triplicate in each experiment and served as the negative control throughout the study. A single aliquot of each spiked armored RNA dilution was used for the further evaluation of two different extraction procedures: the silica column extraction (RNeasy; QIAGEN Inc.), recommended by the developer of each assay, and a high-throughput extraction (MagMax; Ambion, Inc.), shown to be effective for enhancement of the surge capacity during a recent foreign animal disease response (7). Swab fluid was spiked with armored RNA and tested immediately (control value) after storage at 37°C for 24 h, room temperature (20°C to 23°C) for 48 h and 14 days, 4°C for 7 and 14 days, and −20°C for 7 and 14 days. The storage conditions were selected to approximate the extremes of temperature and time conditions that would mimic the range expected for the shipping and handling of proficiency panel samples. Each sample was extracted and tested in triplicate for each time, temperature, and extraction procedure evaluated.

Statistical analysis. The cycle threshold from each qRT-PCR was recorded, and the mean and standard deviation for triplicate samples within experiments and the mean and standard deviation for all experiments for the same extraction procedure were calculated by using standard spreadsheet software (Excel; Microsoft Inc., Redmond, Wash.). Time and temperature stabilities were compared across all experiments by repeated-measures analysis of variance by use of the same spreadsheet software. Interassay reproducibility was recorded as the coefficient of variation (CV), based on the C_T values for all sample replicates tested in the time and temperature study experiments.

RESULTS

Armored RNA was detected over the entire analytic ranges reported for each of the four target viruses by using each of the four extraction protocols, including the phenol-chloroform protocol, the procedures of two spin column kits, and a magnetic bead procedure. The assay-specific limits of detection for the armored RNA target were from 10 to 10^2 target copies for VSV, 10^2 to 10^3 copies for CSFV, and 10^3 to 10^4 copies for FMDV, depending on the extraction procedure used. Equivalent detection limits were obtained by using phenol-chloroform, the magnetic beads, and the prototype spin column (Ambion, Inc.). The alternative spin column (RNeasy; QIAGEN) was less efficient by approximately 1 log unit for each of the four targets evaluated. No overall difference ($P > 0.10$) in the

TABLE 1. Cycle threshold values for all time and temperature stability experiments combined ($n = 8$ experiments)

Assay	Extraction method	Mean (SD) C_T value for the following armored RNA dilution (target copy no./μl):		
		10^{-3} (5×10^6)	10^{-4} (5×10^5)	10^{-5} (5×10^4)
CSFV	Column[a]	26.91 (0.97)	30.44 (1.02)	34.96 (1.44)
	Bead[b]	23.30 (0.37)	27.29 (0.62)	30.57 (0.49)
	Difference	3.61	3.15	4.39
FMDV	Column	31.95 (0.87)	35.64 (1.22)	44.48 (5.65)[c]
	Bead	28.47 (0.98)	32.89 (1.55)	36.57 (1.22)
	Difference	3.48	2.75	7.91
VSV-IND	Column	25.65 (0.97)	28.49 (1.07)	32.49 (2.55)[d]
	Bead	22.22 (0.75)	26.12 (0.90)	28.91 (1.12)
	Difference	3.43	2.37	3.58
VSV-NJ	Column	24.02 (0.72)	27.57 (1.29)	31.65 (2.72)[e]
	Bead	20.44 (0.34)	24.24 (0.62)	27.64 (0.60)
	Difference	3.58	3.33	4.01

[a] RNeasy; Qiagen, Inc.
[b] MagMax; Ambion, Inc.
[c] For FMDV column extraction, C_T values were omitted for six false-negative results obtained at the 10^{-5} dilution.
[d] For VSV-IND column extraction, C_T values were omitted for three false-negative results obtained at the 10^{-5} dilution.
[e] For VSV-NJ column extraction, C_T values were omitted for three false-negative results obtained at the 10^{-5} dilution.

C_T values was detected for any of the time or temperature storage combinations when they were analyzed by extraction procedure. There was, however, a significant difference ($P < 0.01$) between the two extraction techniques used in the stability study, with the bead-based technology providing earlier detection by two to seven cycle thresholds compared to that by the column-based technique for all time and temperature combinations (Table 1). The bead-based extraction method detected all samples at all dilutions used in the stability study, where the column-based procedure had a total of 12 false-negative results among the 72 datum points (17%). All of the column-based false-negative results occurred at the highest dilution (lowest target), with six false-negative findings in the FMDV assay and three false-negative findings in each of the vesicular stomatitis virus assays. The false-negative results by the FMDV assay occurred with the samples stored at 4°C for 7 days ($n = 2$ replicates) and 14 days ($n = 1$ replicate) and at −20°C for 7 days ($n = 3$ replicates). The false-negative results by VSV-IND assay occurred with the control sample ($n = 2$ replicates) and the sample stored at 4°C for 14 days ($n = 1$ replicate), all the false-negative results by the VSV-NJ assay occurred with the initial control sample, which required that a second aliquot be prepared and extracted. CSFV-spiked armored RNA was recovered at all dilutions by both extraction procedures. The coefficient of variation for assay replicates based on the C_T value was less than 7%, regardless of the specific assay and for either the column-based extraction protocol or the bead-based extraction protocol. By use of the column-based extraction protocol, the CVs were 3.8 for CSFV, 6.3 for FMDV, 5.1 for VSV-IND, and 5.4 for VSV-NJ. By use of the bead-based extraction protocol, the CVs were 1.8 for CSFV, 3.8 for FMDV, 3.6 VSV-IND, and 2.13 for VSV-NJ.

DISCUSSION

An armored RNA surrogate capable of being substituted for live virus was developed and evaluated for use as a training and proficiency test tool. For cost efficiency and convenience, targets for each of four foreign animal disease viruses were packaged as a single armored RNA. The custom-made armored RNA was shown to be able to substitute for all four target viruses within the analytic range of the respective qRT-PCR assays when it was spiked into oral swab fluid, a clinical sample matrix appropriate for use with the assays evaluated. The ability to train and test technical proficiency by using representative clinical materials is considered important, particularly in PCR-based diagnostics, where nucleic acids coextracted from host tissues or sample-associated microbes can overwhelm or interfere with both extraction and PCR efficiency. The current study was limited to oral swab fluid specimens; however, it demonstrated the ability to recover the custom-designed armored RNA from a spiked clinical sample matrix. The findings suggest that training and proficiency samples can be prepared to closely mimic diagnostic case material in order to assess not only assay procedures but also the entire chain of sample processing through result interpretation, as appropriate for specific disease agents within a laboratory setting. Because the armored RNA in a clinical sample is less sensitive than native RNA to endogenous ribonucleases, an armored RNA proficiency panel would be expected to be less sensitive to temperature and shipping stresses; so specifically, as a test of the sample handling steps, the surrogate may not detect inefficiencies in maintenance of the cold chain and sample viability. However, after the target RNA is freed from the protective coat protein, the target RNA would again be susceptible to ribonucleases and competing RNA coextracted from clinical materials, mimicking natural testing conditions.

The results of the temperature and time stability measures indicate that a range of ambient temperature and storage conditions did not adversely influence the armored RNA proficiency panel performance. The data indicate that armored RNA can survive at ambient room temperature for 2 weeks with no loss of activity in swab fluid. Although the experiments were not carried to the point of decay, the current findings are

consistent with those of prior reports that armored RNA is stable for 2 and 6 months at 4°C and −20°C, respectively (8).

The differences in qRT-PCR assay design and the resulting efficiency (5) for each of the assays used were consistent with the different detection limits observed with the armored RNA targets in this study. Although the armored RNA contained the same target copy number for each of the four targets at any single dilution, the detection limit was specific to the individual assay, with the VSV assays demonstrating better PCR efficiency than the CSFV and FMDV assays. Based on the CV used to measure interassay variability, armored RNA yielded reproducible results that were consistent with or that exceeded the performance obtained in similar tests with the same four viruses inactivated with binary ethyleneimine and tested by the identical qRT-PCR assay protocols (Tammy Beckham [Plum Island Animal Disease Center, USDA], personal communication).

A statistical difference ($P < 0.01$) was identified when alternate extraction techniques, those with a commercial silica-based column and a commercial magnetic bead kit, were compared. The pattern of false-negative results with the column-based extraction suggests that the failure was due to RNA recovery inherent to the extraction kit at the low target end of the assay rather than to armored RNA decay, as the same technique detected the target in replicates of the samples handled in the same manner and in samples stored at the same temperatures for a longer period of time. The 2- to 7-C_T difference in the levels of detection between the two extraction procedures evaluated approximates a 1- to 2-log-unit difference in virus detection. For silica-based columns, failure may be associated with clogging of the filter pores, sample leakage around the filter disk, or inefficient removal of the RT-PCR inhibitors that may be found in clinical samples. Bead-based technologies, by comparison, have a larger reaction surface which is designed to enhance nucleic acid binding and recovery (9). The efficiency of conversion of the recovered RNA to cDNA during reverse transcription is also significantly affected as the amount of the available target approaches assay detection limits (5), as was observed in this study by greater variability in C_T values and the increased incidence of assay failure for samples containing lower target concentrations. The observation of enhanced assay performance by the use of bead-based extraction warrants further evaluation to confirm similar improvements when each of the four assays evaluated are applied to the live target virus.

The demonstrated performance and ability of armored RNA to act as a surrogate virus will allow PCR standards, assay controls, and training or proficiency samples to be generated, stored for use as needed, and shipped safely as noninfectious reagents. For molecular biology-based assays, where the target agents are considered high risk for national or international distribution, noninfectious and clinically relevant training and proficiency test samples are critically needed for the successful standardization and use of the diagnostic tools. The armored RNA designed here as a surrogate for four foreign animal disease viruses was shown to provide a safe and clinically representative alternative to live virus, chemically inactivated virus, or transcribed RNA as a source of assay-specific RT-PCR positive controls, standards, or training and proficiency samples.

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